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PURIFIED HUMAN BASOPHILS DO NOT GENERATE LTB_4*

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Abstract—We investigated the release of the 5-lipoxygenase derivatives of arachidonic acid (AA) in purified human basophils and compared them with similar results obtained in the human lung mast cell. We have shown that purified basophils (average purity = 51 ± 6%) challenged with 0.1 μg/ml anti-IgE released histamine (35 ± 9%), and LTC_4 (32 ± 10 ng/10^6 cells) but failed to release measurable quantities of immunoreactive LTB_4. In contrast, the non-specific stimulus, A23187, caused the release of histamine and both LTC_4 (279 ± 95 ng/10^6 cells) and LTB_4 (148 ± 41 ng/10^6 cells). Closer analysis of the data revealed an inverse relationship between the levels of LTB_4 released and the purity of the basophils, strongly suggesting that the contaminating monocytes were responsible for LTB_4 synthesis. Purified human lung mast cells have been shown to release 6 ng of immunoreactive LTB_4/10^6 cells, indicating that basophils release significantly less LTB_4 following an IgE-mediated challenge. In a series of experiments using highly purified basophils prelabeled with [^{3}H]AA, we demonstrated that exposure to 0.1 μg/ml anti-IgE led to the release of [^{3}H]LTC_4, with no detectable [^{3}H]LTB_4, whereas exposure to 1.0 μg/ml A23187 caused the release of [^{3}H]LTC_4 and smaller quantities of [^{3}H]LTB_4, [^{3}H]LTD_4, and [^{3}H]LTE_4. We failed to detect any [^{3}H]LTB_4 in the cell pellet following challenge with either anti-IgE or A23187, indicating that LTB_4 was not synthesized and retained within the cell pellet. Finally, we found that exogenously added [^{3}H]LTB_4 was not metabolized, either by basophils alone or by basophils stimulated with anti-IgE (0.1 μg/ml).

The dihydroxy lipoxygenase derivative of arachidonic acid known as leukotriene B_4 (LTB_4) was first identified by Borgeat and Samuelsson in 1979 [1]. Considerable interest has focused on this compound because of its possible role in allergic and inflammatory disorders. LTB_4 is a potent chemotactic and chemokinetic agent for both neutrophils [2] and eosinophils [3] and, in combination with prostaglandin E_2 (PGE_2), causes increased vascular permeability [4]. Unlike the sulfidopeptide leukotrienes LTC_4, LTD_4 and LTE_4, which are potent bronchoconstrictors [5], LTB_4 has much less spasmogenic activity and appears to act via the release of thromboxane A_2 [6].

LTB_4 has been identified in a range of inflammatory conditions including the sputum of patients with cystic fibrosis [7], the synovial fluid of patients with gout [8], rheumatoid arthritis and spondyloarthritis [9], in the skin and in skin chambers of patients with psoriasis [10], the mucosa of patients with inflammatory bowel disease [11], and also the nasal washings obtained from patients with allergic rhinitis [12]. These findings strongly suggest that LTB_4 plays a role in the pathophysiology of inflammatory diseases; however, its precise role has not been delineated in part because of the lack of specific antagonists and the rapid *in vivo* metabolism of LTB_4 via ω-oxidation [13, 14].

One possible role for LTB_4 in the inflammatory process is in the amplification of an initial signal. It has been suggested that the event which initiates a late phase reaction (LPR), the recurrence of allergic symptoms some 4–12 hr after the initial response, is the degranulation of mast cells [15, 16]. The mast cell mediators then lead either directly or indirectly to the recruitment of granulocytes and the amplification of the response. The discovery that human lung mast cells release small amounts of LTB_4 *in vitro* [17] further suggests that mast cell degranulation could lead directly to the accumulation of neutrophils and eosinophils at the initial lesion. We have recently obtained circumstantial evidence linking the basophil and not the mast cell with the initiation of the LPR *in vivo* [18]. We have also obtained evidence that basophils respond to IgE-dependent factor(s) present in skin blister fluids collected during the LPR, whereas lung mast cells are largely unresponsive [19]. These results suggest that basophil-derived mediators may be pivotal in the pathogenesis of the LPR and raised the question of whether basophils, like mast cells, could synthesize LTB_4 and hence provide a mechanism for the amplification of the initial IgE-dependent release of mediators. We therefore set out to investigate the possiblity that immunologic stimulation of basophils could cause the

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release of LTB_4. We used both isotopic methods and specific radioimmunoassays.

MATERIALS AND METHODS

The following were purchased: piperazine-*N,N'*-bis-2-ethanesulfonic acid (PIPES) and heparin (Sigma, St. Louis, MO); calcium ionophore A23187 (CalBiochem, Los Angeles, CA); crystallized human serum albumin (Miles Laboratories, Elkhart, IN); RPMI 1640 with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and fetal calf serum (Gibco, Grand Island, NY); Percoll, Sepharose CL-4B, and Sepharose CL-6MB (Pharmacia Fine Chemicals, Piscataway, NJ); arachidonic acid and phospholipid standards (Supelco, Bell Parke, PA); HPLC grade solvents (Burdick & Jackson, Bodman Chemicals, Media, PA); [^{3}H]arachidonic acid ([^{3}H]AA) (84 Ci/mmol), [^{3}H]LTC_4 (20–60 Ci/mmol) and [^{3}H]LTB_4 (80–120 Ci/mmol) (New England Nuclear, Boston, MA). The leukotriene standards and antibodies were obtained from the following people: leukotrienes B_4, C_4, D_4, and E_4 (Dr. Joshua Rokach, Merck Frosst, Canada); anti-leukotriene B_4 (Dr. John Humes, Merck Institute); and anti-leukotriene C_4 (Dr. Edward Hayes, Merck Institute).

Buffers. PIPES-albumin-glucose (PAG) buffer contains 25 mM PIPES, 100 mM NaCl, 5 mM KCl, 0.1% glucose and 0.003% human serum albumin. Release experiments were carried out in PAG containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PAGCM). Basophil purification and labeling were carried out in RPMI 1640 supplemented with 4 mM EDTA, 5% fetal calf serum and 10 μg/ml heparin.

Basophil purification. Basophils were purified using the method of MacGlashan and Lichtenstein [20], which yielded preparations containing 31–92% basophils with monocytes and lymphocytes accounting for the remaining cells.

Basophil labeling. Basophils ($2–5 \times 10^6$ cells/ml) were incubated with 25 μCi of [^{3}H]AA for 1 hr at 37°. The cells were then washed extensively in PAG to remove the non-specifically bound [^{3}H]AA.

Challenge. Labeled basophils (1×10^6 cells/ml) were resuspended in PAGCM and challenged with either 44% D_2O, 0.1 μg/ml anti-IgE, or 1.0 μg/ml of the calcium ionophore A23187. Doses were chosen to give the maximum non-cytolytic mediator for each stimulus. The cells were incubated at 37° for 45 min; the reaction was then halted by centrifugation (300 *g*, 2 min) and the supernatant fraction was recovered.

Histamine release assay. Aliquots of cell suspension and supernatant fraction were added to tubes containing 0.8 ml PAG and 0.2 ml 8% $HClO_4$. Histamine release was measured using the automated fluorometric technique of Siraganian [21]. Spontaneous release (usually 5–8%) was subtracted from all experimental values.

Lipid techniques. Supernatant fractions from control and stimulated basophils were extracted with ethanol and chloroform as previously described [22]; extraction efficiencies were 77 ± 3%, 88 ± 1% and 76 ± 6% for [^{3}H]LTC_4, [^{3}H]LTB_4 and [^{3}H]AA respectively. The organic solvent was evaporated under nitrogen, and the residue was dissolved in 0.1% acetate buffer, pH 4.6: methanol (1:2, v/v). HPLC was performed on a Beckman model 342M liquid chromatograph, which includes model 100A and 110A pumps, 421 systems controller, 210 sample injector with 50 μl injection loop, 155–40 UV visible-wavelength detector and a C-RIA plotter integrator. An ultrasphere ODS (C_{18}) column (4.5 mm × 25 cm) and a Supelco cartridge C_{18} pre-column were used for the separation of the leukotrienes [23].

Radioimmunoassays. Radioimmunoassays for LTC_4 [24] and LTB_4 [25] were performed as described earlier, using dextran-coated charcoal as the separation technique. Cross-reactivities for other lipid mediators were as previously described [24, 25].

Table 1. Release of mediators from purified basophils following exposure to anti-IgE

% Basophil purity	% Histamine release	LTC_4 release (ng/10^6 cells)	LTB_4 release (ng/10^6 cells)
31	28.5	17.5	<6.3
37	18.2	22.8	1.6
57	65.1	73.0	<6.3
63	50.5	32.4	<3.15
67	12.3	13.3	<1.5
51 ± 6.4	34.9 ± 8.9	31.8 ± 9.6	Undetectable

Five preparations of partially purified basophils were challenged with 0.1 μg/ml anti-IgE, and the release of histamine, LTC_4 and LTB_4 was measured. The results are summarized as mean ± SE at the bottom of the table.

RESULTS

Purified human basophils (average purity 51 ± 6%) were challenged with 0.1 μg/ml anti-IgE (N = 5), and mediator release was measured. These results are summarized in Table 1. Histamine release ranged from 12.3 to 65.1% and was accompanied by the release of immunoreactive LTC_4 in each donor. These results reflect the activation of the basophils and the release of both preformed and newly synthesized mediators. No immunoreactive LTB_4 could be detected using the specific radioimmunoassay in four out of five experiments; however, in one experiment we were able to detect a small amount of $iLTB_4$. As this experiment involved one of the less pure basophil preparations, the amount of $iLTB_4$ measured was equal to the limit of detection in the radioimmunoassay (1.5 ng/10^6 cells). We suggest that this does not represent significant release of $iLTB_4$ from immunologically challenged cells. Similar results (no measurable LTB_4) were obtained using 44% D_2O (N = 3) (data not shown). Histamine release was 38 ± 12% and was accompanied by the release of LTC_4 (15 ± 6 ng/10^6 basophils). The results obtained with the nonspecific stimulus A23187 reflects the capacity of the contaminating cells, principally monocytes, to generate LTB_4 [26, 27] (see Table 2). Histamine release was 58 ± 6%, whilst release of both the lipoxygenase products was greatly increased. LTC_4 release was 279 ± 95 ng/10^6 basophils, almost a 10-fold increase over the immunologically challenged

Table 2. Release of leukotrienes from partially purified basophils following exposure to the calcium ionophore, A23187

% Basophil purity	LTC_4 release ($ng/10^6$ cells)	LTB_4 release ($ng/10^6$ cells)
31	80	248
37	483	110
67	274	86
45 ± 9	279 ± 95	148 ± 41

Three preparations of basophils were challenged with 1.0 μg/ml A23187, and the release of LTC_4 and LTB_4 was measured. The results are summarized as mean ± SE at the bottom of the table.

cells, and the LTB_4 release was 148 ± 41 ng immunoreactive LTB/10^6 basophils.

In an earlier series of experiments, our laboratory [17, 28] demonstrated that purified human lung mast cells challenged with anti-IgE released histamine, approximately 50 $ng/10^6$ cells of both LTC_4 and PGD_2, and 6 $ng/10^6$ cells of immunoreactive LTB_4. The level of LTC_4 release is thus comparable in the two cells types, whereas the amount of LTB_4 produced by human basophils is substantially below that liberated by human lung mast cells.

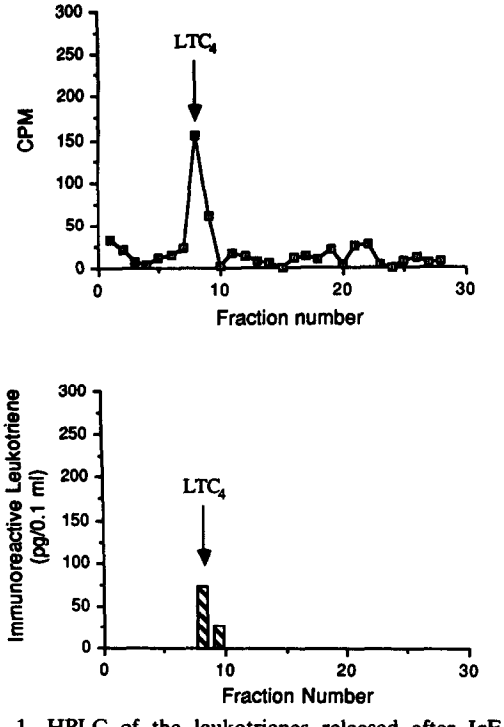

Fig. 1. HPLC of the leukotrienes released after IgE-mediated challenge. Purified basophils were prelabeled with [^{3}H]AA, washed and challenged with 0.1 μg/ml anti-IgE. The supernatant fraction was extracted and the leukotrienes were fractionated by HPLC. The upper panel shows a single peak of radioactivity corresponding to LTC_4 (N = 4), and the lower panel shows a single peak of immunoreactive LTC_4 (N = 2). There was no peak corresponding to [^{3}H]LTB_4 present, and no immunoreactive LTB_4 was detected.

We next turned to isotopic methods to investigate LTB_4 production by purified human basophils. Basophils (average purity 79 ± 2%) labeled with [^{3}H]AA were challenged with 0.1 μg/ml anti-IgE and 1.0 μg/ml A23187; the lipid mediators were extracted and analyzed by HPLC using a methanol/acetate buffer system which is routinely used for leukotriene separation.

Unstimulated cells, exposed only to PAGCM, released no detectable leukotrienes, and the background counts were subtracted from subsequent experiments to give net release. The cells challenged with anti-IgE showed a peak of radioactivity corresponding to the LTC_4 standard (Fig. 1, upper panel); there was no peak corresponding to either LTB_4 or any of its isomers. In two experiments analyzed without the presence of internal standards, radioimmunoassay of the fractions demonstrated a peak of immunoreactive LTC_4 and failed to detect any immunoreactive LTB_4 (see Fig. 1, lower panel). We also failed to detect either LTD_4 or LTE_4, confirming earlier findings that purified basophils do not metabolize LTC_4 [29].

A nonspecific challenge with A23187 led to the release of [^{3}H]LTC_4 and smaller quantities of [^{3}H]LTB_4, [^{3}H]LTD_4 and [^{3}H]LTE_4 (Fig. 2, upper panel). RIA with an LTC_4-specific antibody confirmed the presence of LTC_4 and LTD_4. Due to the low cross-reactivity between the antibody and LTE_4 (10–12%), we were unable to detect any LTE_4. A second RIA with an LTB_4-specific antibody demonstrated that the remaining peak was LTB_4 (see Fig. 2, lower panel). These experiments confirm our earlier findings that purified human basophils do not produce LTB_4 in response to an immunologic challenge, though at least some of the cells present are capable of responding to a nonspecific challenge. However, because LTB_4 generation is generally entirely intracellular, it has been suggested that under some conditions LTB_4 may be produced during an immunologic challenge but not released [30]. To investigate this possibility, we subjected the lipids remaining in the cell pellet following stimulation to HPLC analysis (data not shown). Neither anti-IgE nor A23187 stimulation led to the intracellular production of [^{3}H]LTB_4 or [^{3}H]LTC_4, indicating that all the leukotrienes produced are released.

The remaining possibility, that LTB_4 was released by basophils and then rapidly metabolized, either by ω-oxidation or other routes, was finally investigated. Purified human basophils (average purity = 61%, N = 2) and buffy coat cells (where basophils accounted for only 1–2% of total cells, N = 2) were incubated with 10^{-7} M [^{3}H]LTB_4 at 37° for up to 45 min either alone or in the presence of 0.1 μg/ml anti-IgE. Aliquots were removed at 0, 5, 15, and 45 min, and the lipids were analyzed by HPLC. The results for the purified and impure cells were the same (data not shown); approximately 80% of the radioactivity co-eluted with an authentic LTB_4 standard, and the remaining 20%, presumably an LTB_4 isomer, was found in a second peak. The proportions of LTB_4 and isomer remained unchanged over the 30-min incubation. Addition of anti-IgE did not

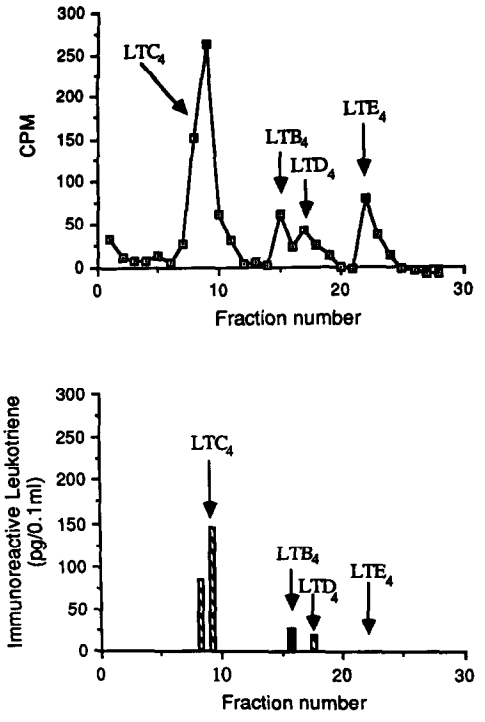

Fig. 2. HPLC of the leukotrienes released after A23187 challenge. Purified basophils were prelabeled with [^{3}H]AA, washed and challenged with 1.0 μg/ml of the calcium ionophore A23187. The supernatant fraction was extracted, and the leukotrienes were fractionated by HPLC. The figure in the upper panel demonstrates that the principal product was [^{3}H]LTC_4, with smaller quantities of [^{3}H]LTB_4, [^{3}H]LTD_4, and [^{3}H]LTE_4 (N = 4). Radioimmunoassay of the fractions, shown in the lower panel, confirmed the presence of LTC_4, LTB_4, and LTD_4, but failed to detect LTE_4 due to the low cross-reactivity of our antibody.

affect the distribution of the label despite the release of 32 ± 8% of the available histamine.

DISCUSSION

The leukotrienes produced by purified human basophils were investigated extensively and we were unable to obtain any evidence that LTB_4 is produced in response to an IgE-mediated challenge. Using a specific radioimmunoassay for LTB_4, we demonstrated that measurable amounts of immunoreactive LTB_4 were released only in response to a nonspecific stimulus, such as A23187. Similar experiments with purified human lung mast cells exposed to anti-IgE revealed the release of approximately 6 ng of immunoreactive LTB_4 per 10^6 cells. The limits of detection in the immunoassays were between 3.2 and 1.5 ng of $iLTB_4/10^6$ cells, indicating that purified human basophils release substantially less $iLTB_4$ than the lung mast cells.

These results were confirmed using isotopic methods. Basophils labeled with [^{3}H]AA release [^{3}H]LTC_4, [^{3}H]5-hydroxyecosatetraenoic acid, unmetabolized [^{3}H]AA and an unidentified prostanoid following anti-IgE challenge [31], but there is no evidence that either [^{3}H]LTB_4 or any nonenzymatically produced isomers of LTB_4 are produced. In contrast, HPLC analysis of the metabolites released after nonspecific stimulation revealed [^{3}H]LTC_4 as the principal metabolite with lesser amounts of [^{3}H]LTB_4, [^{3}H]LTD_4, and [^{3}H]LTE_4. The identity of the leukotrienes was confirmed using two specific radioimmunoassays. Experiments with [^{3}H]AA-labeled human lung mast cells revealed that 30% of the immunoreactive LTB_4 produced by these cells was the biologically active LTB_4 isomer. Therefore, we estimate that purified human lung mast cells produce 2 ng/10^6 cells [17, 28].

Analysis of the lipids remaining in the cell pellet after an anti-IgE challenge failed to reveal the presence of any leukotrienes, suggesting that all the arachidonic acid metabolites are released from the cell during the incubation period. Finally, basophils, either purified or an impure buffy coat preparation, failed to metabolize [^{3}H]LTB_4 over a 45-min incubation period even when the cells were stimulated with anti-IgE. Thus, we have been unable to find any evidence that purified human basophils release LTB_4 in response to an IgE-mediated challenge. The use of the nonspecific stimulus A23187 clearly demonstrates that the cells present even in purified basophil preparations are capable of producing large quantitites of LTB_4, which accompanies the release of histamine and LTC_4. It is not possible to state unequivocally that all the LTB_4 detected in these cases is produced by monocytes rather than the basophils, but the available evidence suggests that it is the contaminating cells which are responsible for LTB_4 synthesis. This capacity of monocytes to produce large quantities of LTB_4 should not be overlooked when results obtained from mixed leukocytes are interpreted.

These experiments also serve to reiterate the profound differences between basophils and mast cells. Whilst the two cell types release similar levels of LTC_4, the mast cells release small but pharmacologically significant amounts of LTB_4 and basophils fail to produce any detectable levels of LTB_4. This work would appear to exclude a direct role for the basophil in the amplification of an allergic response via LTB_4 production but does not rule out important roles for either the basophil or LTB_4 in a wide variety of allergic and inflammatory conditions.

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